Endothelial Nitric Oxide Synthase Gene Variants and Primary Open-Angle Glaucoma: Interactions with Sex and Postmenopausal Hormone Use

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Purpose. To evaluate the association between the nitric oxide synthase gene (*NOS3*) variants and primary open-angle glaucoma (POAG).

Methods. Two functional single-nucleotide polymorphisms (SNPs) (T−786C: rs2070744; Glu298Asp: rs1799983) and three tagging SNPs (rs7830, rs3918188, and rs1800779) were evaluated in a nested case–control study from the Nurses' Health Study (1980–2002) and the Health Professionals' Follow-up Study (1986–2002). Participants were aged ≥40 years and Caucasian. Included were 527 incident cases and 1543 controls, matched by cohort, age, and eye examination at the matched cases' diagnosis dates. Cohort-specific relative risks (RR) were estimated by using multivariable conditional logistic regression and were pooled with meta-analysis.

Results. No *NOS3* polymorphism was significantly associated with overall POAG. For high-tension POAG (HTPOAG), rs3918188 was significantly inversely associated among the women (AA versus CC genotype: RR = 0.48; 95% CI, 0.28–0.82) but not among the men (*P*-heterogeneity by sex = 0.02). The minor alleles of T −786C and rs1800779 showed positive association with high-tension POAG (*P*-trend < 0.02) in the women only, but *P*-heterogeneity was not significant. In the women, four of the five *NOS3* SNPs showed significant interactions with postmenopausal hormone (PMH) use in relation to HTPOAG: for example, among the women with the TT genotype in T −786C, PMH use was inversely associated (RR = 0.41; 95% CI, 0.22–0.76), but among carriers of the minor allele, use of PMH was not associated.

Conclusions. Interactions were observed between *NOS3* SNPs and female sex and postmenopausal hormone use in the women in relation to HTPOAG. These findings should be confirmed in different racial/ethnic groups. (*Invest Ophthalmol Vis Sci.* 2010;51:971–979) DOI:10.1167/iovs.09-4266

Nitric oxide synthase (NOS) has three genetically distinct isoforms: neuronal NOS encoded by *NOS1* (12q24-q24.3),[1] macrophage-derived isoforms encoded by genes on chromosome 17 (*NOS2A*, *-2B*, and *-2C*),[2] and an endothelium-derived isoform encoded by *NOS3* (7q36).[3] Endothelial NOS is constitutively expressed in the human outflow pathway and ciliary muscle where it may modulate outflow facility.[4] It is also expressed on all vascular endothelial cells, including those in the optic nerve pedicle.[5] Nitric oxide (NO), formed by NOS in endothelial cells, mediates vasodilation in response to acetylcholine and thus is important in mediating vascular tone and modulating blood flow to the optic nerve.[6]

Endothelial dysfunction may play a key role in POAG pathogenesis, and it can be detected in patients with early disease.[7,8] Patients with POAG demonstrate abnormal vascular regulation in various ocular tissue beds[7–14] and abnormal brachial artery vasodilation in response to acetylcholine.[8,15] Also, an interventional study using an NOS inhibitor found differences in ocular blood flow response between patients with POAG and controls, implicating the L-arginine/nitric oxide system.[16] Thus, alterations in *NOS3* activity produced either by genetic variation or by environmental influences could play a role in the pathogenesis of glaucoma.

Recent genetic studies of *NOS3* also support the involvement of endothelial NOS expression and NO synthesis. Polymorphisms in *NOS3* or in the 5′ upstream untranslated region have been associated with high-tension POAG[17] and POAG with migraine.[18] Studies to date have been relatively small (<200 cases) and their results have been inconsistent. In addition, researchers have not examined interactions between *NOS3* and environment factors in POAG. For example, estrogen is known to upregulate *NOS3*,[19] and later age at menopause[20,21] and the use of postmenopausal hormones (PMHs) is inversely associated with the risk of POAG.[22] However, no investigators have evaluated the interaction between the individual's sex, reproductive aging, and *NOS3* in relation to POAG.

Therefore, we evaluated the association between five single-nucleotide polymorphisms (SNPs) of *NOS3* and POAG in a matched, nested case–control study with 527 cases and 1543 control subjects selected from participants in the Nurses' Health Study and Health Professionals' Follow-up Study. We also examined whether there were interactions with age at menopause and PMH use and POAG among the postmenopausal women.

Methods

Study Population

We conducted a nested case–control study within the Nurses' Health Study (NHS) and Health Professionals Follow-up Study (HPFS) cohorts.

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The NHS was established in the United States in 1976, when 121,700 registered nurses between the ages of 30 and 55 years returned a questionnaire on health-related exposures.[23] The HPFS started in 1986 with 51,529 U.S. male health professionals aged 40 to 75 years who responded to a similar mailed health questionnaire. Participants were followed up with biennial questionnaires to update information on lifestyle factors and newly diagnosed illnesses, such as glaucoma.[24] Follow-up rates were high (>95% of the total possible person time through 2002). The Human Research Committees of Brigham & Women's Hospital, Massachusetts Eye and Ear Infirmary, and the Harvard School of Public Health approved the study. Our research adhered to the tenets of the Declaration of Helsinki.

Blood and Buccal Sample Collection

Blood samples were collected from 32,826 (27%) women between 1989 and 1990 and from 18,225 (35%) men between 1993 and 1995. Buccal cell samples were collected between 2001 and 2004 from 29,684 of the women who had not provided a blood sample. Follow-up was >95% in both of these subcohorts.

Blood samples, collected with heparin sodium used as an anticoagulant, were returned within 26 hours of being obtained, immediately centrifuged; aliquoted into plasma, red blood cells, and buffy coat components; and stored in liquid nitrogen freezers. All buccal cell samples were collected in a single "swish-and-spit" procedure in which subjects were provided a small bottle of mouthwash and a small cup with a screw-on cap and were asked to swish the mouthwash and then spit into the cup.[25] Samples were processed within a week of receipt.

Case and Control Ascertainment

We ascertained POAG cases biennially, in a three-step process. First, in each questionnaire, we asked whether eye examinations had been performed and whether a diagnosis of glaucoma had been given. Second, we sought permission to retrieve the ophthalmic records of participants who reported glaucoma. We contacted the diagnosing physician for copies of all visual field (VF) tests to date and for the completion of a glaucoma questionnaire regarding maximum IOP, the status of the filtration apparatus, optic nerve structural information, prior ophthalmic surgery, and any VF loss. We allowed physicians to send relevant medical records in lieu of completing the questionnaire. To determine case status, a glaucoma specialist (LRP) evaluated all the ophthalmic information from questionnaires/medical records and VF results in a standardized manner.

Only participants with "definite" or "probable" POAG were included. From participants with definite POAG, we required documentation of gonioscopy showing that angles were not occludable in either eye, slit lamp biomicroscopy showing no indication in either eye of pigment dispersion syndrome, uveitis, exfoliation syndrome, trauma, or rubeosis, and two or more reliable VF results showing reproducible defects that were consistent with glaucoma. For probable POAG cases, the slit lamp examination and VF criteria were also required, but documentation of pupil dilation without subsequent adverse events was accepted instead of gonioscopy. Among cases, >70% met the criteria for definite POAG. For VF results, there was no requirement regarding the type of perimetry performed; however, in 95% of cases, full static threshold testing was completed, and in <1% of cases, kinetic VF tests were used. For static threshold or suprathreshold testing, we considered the VF result to be reliable if the fixation loss rate was ≤33%, the false-positive rate was ≤20%, and the false-negative rate was ≤20%. For kinetic VFs, we consider the results reliable unless there was notation by the examiner to the contrary.

We included 527 glaucoma cases and 1543 control subjects (373 NHS cases and 1082 controls; 154 HPFS cases and 461 controls), aged ≥40 years and Caucasian (<20 cases were Latino). The control subjects were matched on sex, type of DNA sample (blood or cheek cell), year of birth and ethnicity (Latino or not), and they were required to have had an eye examination at the same period as the diagnosis date of the matched case. Approximately three control subjects were matched to each case, by using incidence density sampling.

Genotyping

Two functional single-nucleotide polymorphisms (SNPs) (T −786C: rs2070744, and Glu298Asp: rs1799983) and three tagging SNPs (rs1800779, rs3918188, and rs7830) were genotyped. The tagging SNPs corresponded to the three *NOS3* linkage disequilibrium (LD) blocks (Fig. 1) and were selected by using Haploview (ver. 4.1) according to the HapMap data (release 22) from the CEU population.[26] The minimum minor allele frequency for checking markers was set to 0.01. Three tagging SNPs (rs1800779, rs3918188, and rs7830) were selected to capture the majority (88%) of alleles at $r^2 > 0.8$ across the whole gene, including the 5′ and 3′ untranslated regions.

For DNA extraction, 50 μL of buffy coat or 20 μL of buccal cells were diluted with 150 μL of PBS and processed (QIAmp 96 spin blood kit protocol; Qiagen, Inc., Chatsworth, CA). A quantitative PCR approach (TaqMan Assay; Applied Biosystems, Inc. [ABI], Foster City, CA) was used for genotyping, according to the manufacturer's instructions. The RT-PCR amplification of genomic DNA was performed in 96-well plates with a sequence-detection system (Prism 7000; ABI). The thermal cycler (model 2720; ABI) was set at the following parameters: 50°C for 2 minutes, 95°C for 10 minutes, 92°C for 15 seconds, and 58°C for 1 minute, for 60 cycles. The genotyping success rate was >90% for all five SNPs included in the study. Plates that passed quality control measures (including Hardy-Weinberg equilibrium tests) were included, and in 5% of samples that underwent repeat genotyping, there was >95% concordance on genotyping calls.

Assessment of Menopausal Status, Age at Menopause, and Postmenopausal Hormone Use among Women

Starting in 1976, we asked NHS participants to update the information on their reproductive histories. We asked participants if they had entered menopause, the age of onset, and the type of menopause experienced (natural, radiation-induced, or surgical). Beginning in 1980, for surgically induced menopause, we inquired about the nature of the surgery (whether one or both ovaries were removed and whether the uterus was also removed). We also asked participants whether they had taken PMHs, and if so, for how long and what type of hormone was used (unopposed oral-conjugated estrogen, estrogen with progesterone, and other estrogens). In validation studies, the self-reported information on reproductive history given by the NHS participants has been found to be highly accurate.[27–30]

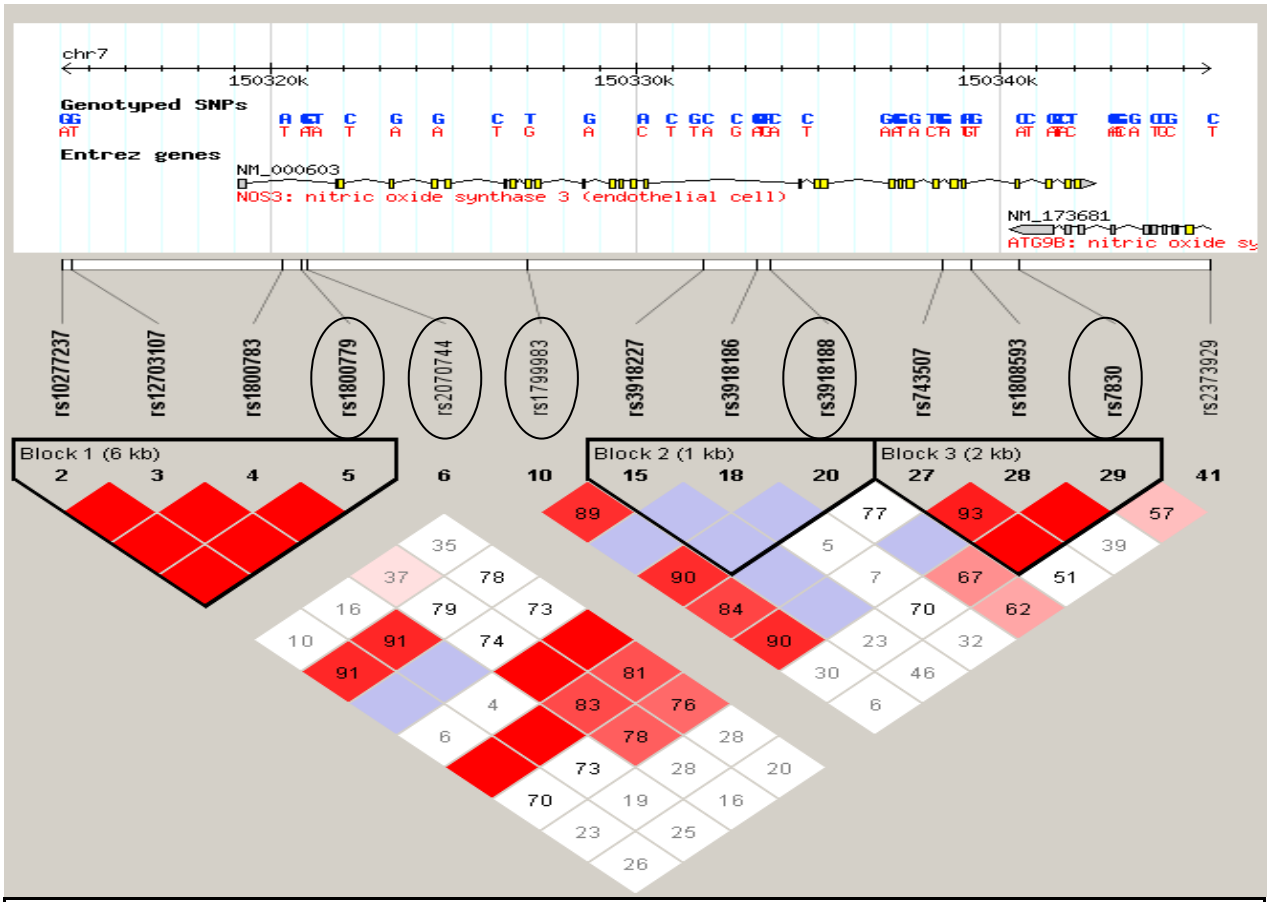
Statistical Methods

We analyzed the cohort-specific data separately with conditional logistic regression, adjusting for potential confounders. Then, we pooled the results using meta-analytic methods, incorporating random effects.[31] $P < 0.05$ was considered statistically significant (SAS, ver. 9.1.3; SAS, Cary, NC).

Information on potential confounders was obtained from the biennial questionnaires and was updated through the questionnaire completed immediately before the date of the diagnosis of the index case. Potential confounders were family history of glaucoma, body mass index (<22, 22–23.9, 24–25.9, 26–27.9, 28–29.9, or ≥30 kg/m^2), smoking status (current, past, or never), physical activity (quartiles of activity intensity/day), self-reported history (yes/no) of hypertension and diabetes, cumulatively updated alcohol intake (0, 1–9, 10–19, 20–20, or ≥30 g/d), and cumulatively updated caffeine intake (0–149, 150–299, 300–449, 450–600, or ≥600 g/d).

Secondary Analyses

In secondary analyses, we separately analyzed the risk of high-tension POAG, defined as maximum IOP ≥ 22 mm Hg before visual field loss



Haplotype	Tagging SNP rs1800779	Functional SNP rs2070744 (-786C/T)	Functional SNP rs1799983 (Glu298Asp)	Tagging SNP rs3918188	Tagging SNP rs7830	Cases %	Controls %	All POAG*	High Tension POAG*	Normal Tension POAG*
	(Promoter)	(Promoter)	(Exon 7)	(Intron)	(Intron)			RR (95% CI)	RR (95% CI)	RR (95% CI)
1	A	T	G	**A****	C	30.3	32.2	1.00	1.00	1.00
2	**G**	**C**	**T**	C	C	23.6	23.6	1.04 (0.84,1.29)	1.19 (0.73,1.95)	0.79 (0.50,1.25)
3	**G**	**C**	G	C	**A**	15.7	15.4	1.06 (0.76,1.49)	1.45 (0.99,2.12)	0.77 (0.48,1.22)
4	A	T	G	C	**A**	13.1	13.8	0.96 (0.73,1.26)	1.06 (0.76,1.48)	0.36 (0.09,1.47)
5	A	T	**T**	C	C	9.0	7.4	1.43 (1.01,2.02)	1.68 (1.09,2.58)	0.97 (0.36,2.57)
6	A	T	G	C	C	8.3	7.6	1.05 (0.57,1.93)	1.12 (0.30,4.17)†	1.22 (0.63,2.35)

* Likelihood ratio test of haplotype effect for overall POAG: NHS, p=0.28; HPFS, p=0.80; for high tension POAG: NHS, p=0.004; HPFS, p=0.68; for normal tension POAG: NHS, p=0.28; HPFS, p=0.02.

** Bold denotes variant

† P-heterogeneity between men and women was significant: p=0.007. In the NHS, the RR = 2.10 (95% CI=1.28, 3.46), and in the HPFS, the RR = 0.55 (95% CI, 0.24, 1.26)

FIGURE 1. Linkage disequilibrium between *NOS3* SNPs using the Haploview software (version 4.1)[26] expressed in terms of r^2 and haplotype analyses of *NOS3* gene polymorphisms.

(67.5% of all POAG cases), and normal-tension POAG, defined as those with maximum IOP < 22 mm Hg before visual field loss. Polytomous logistic regression was used to test whether an individual SNP was related differently to high-tension compared with normal-tension glaucoma.[32]

We also conducted haplotype analyses using genotyping data from all five SNPs. Haplotype frequencies of the *NOS3* gene were estimated (PROC Haplotype; SAS ver. 9.1.3; SAS).

Effect Modification with Age at Menopause and PMH Use

Among the postmenopausal women only, we evaluated interactions between the *NOS3* SNPs and age at menopause (≥54 years vs. <54 years; 54 years was the median age at menopause) and between *NOS3* SNPs and the use of PMHs (current use versus nonuse).

Table 1. Characteristics of Cases of POAG and Their Matched Controls as of the Diagnosis Date

Characteristics	Cases	Controls
Age, mean, y		
Women	64.2	64.2
Men	67.1	67.1
Family history of glaucoma, %		
Women	35.4	12.5
Men	30.1	11.3
Diabetes, %		
Women	7.7	5.2
Men	8.0	5.5
Obesity (Body mass index ≥ 30 kg/m2, %)		
Women	13.7	15.2
Men	9.5	10.9
Hypertension, %		
Women	37.4	39.4
Men	37.7	34.8
30+ pack years of smoking, %		
Women	17.4	18.6
Men	19.4	23.4
Caffeine (mean, mg/day)		
Women	310	307
Men	223	226
Alcohol intake (mean, g/day)		
Women	6.0	6.2
Men	11.3	12.9
Reported eye examinations, *n**		
Women	3.1	3.2
Men	3.2	3.2
Current postmenopausal hormone use		
Women	39.6	42.4
Men	—	—

* Eye examinations have been asked every 2 years, seven times from 1990 to 2002; the number represents the number of eye examinations reported as of the diagnosis date of the index case in matched case–control sets.

To evaluate effect modification, we tested the significance of the pooled estimates of the interaction terms from the multivariate conditional logistic regression models.

Results

The POAG cases and matched control subjects were similar in their characteristics as of the index case's date of diagnosis (Table 1). All cases and controls were Caucasian and matched for age. As expected, cases had a higher frequency of a family history of glaucoma. In addition, they had a higher frequency of diabetes; however, they were somewhat less likely to be obese, drink alcohol, or smoke. Among the women, the cases were less likely to be current PMH users.

Association with *NOS3* SNPs

The five *NOS3* polymorphisms were not associated with overall POAG (Table 2), and the associations were not significantly different between the men and the women. For example, for the T −786C polymorphism, compared with the TT genotype, the pooled RR was 1.05 (95% CI, 0.83–1.34) for the CT genotype and 1.00 (95% CI, 0.64–1.55) for the CC genotype. Also, for the Glu298Asp polymorphism, compared with the GG genotype, the pooled RR was 1.04 (95% CI, 0.82–1.32) for GT and 1.33 (95% CI, 0.94–1.86) for TT.

In secondary analyses in which we evaluated subtypes of POAG defined by highest IOP at diagnosis, we found one SNP that was associated with high-tension glaucoma (Table 3): for the tagging SNP rs1800779, the RR was 1.13 (95% CI, 0.85–1.52) for the AG genotype and 1.58 (95% CI, 0.97–2.55) for the GG genotype, with a significant trend (P = 0.02). For this SNP, although the P-heterogeneity for sex effect was not significant, among the women, the association was stronger (RR = 1.87, 95% CI, 1.19–2.94 for the comparison between the GG versus AA genotype, P-trend = 0.01). Similarly, the associations of other SNPs appeared to be stronger in the women, although the P-heterogeneity for sex was not significant for most of the SNPs. In the one SNP (rs3918188) that showed significantly different association by sex (P-heterogeneity = 0.02), the RR for the AA compared with the CC genotype was 0.48 (95% CI, 0.28–0.82) among the women (P-trend = 0.0008) but 1.48 (95% CI, 0.77–2.84) among the men (P-trend = 0.61).

In relation to normal-tension glaucoma, associations were observed with the functional promoter SNP (Table 4). The pooled RR for the CC homozygote was 0.44 (95% CI, 0.22–0.87) and P-trend = 0.03. The T minor allele for the Glu298Asp polymorphism was adversely associated (TT versus GG: RR = 1.81; 95% CI, 0.93–3.52) but the association was not significant. Other SNPs were not associated with normal-tension glaucoma.

For the SNP rs3918188, that showed significantly different associations between the men and the women, the associations with high- and normal-tension glaucoma were also significantly different (P = 0.02) among the women. Similarly, the associations with the two subtypes of POAG were also different for the tagging SNP rs1800779 as well as the T −786C polymorphism (P = 0.04 and P = 0.02, respectively) among women.

There were no haplotype effects observed for overall POAG ($P > 0.20$ in both the men and the women; Fig. 1). However, in the women, we observed significant haplotype effects in relation to high-tension glaucoma (likelihood ratio test P = 0.004), whereas in the men we did not (likelihood ratio test P = 0.68). In the women, the significant increase in risk with haplotype 6 (RR = 2.10; 95% CI, 1.28–3.46) compared with haplotype 1 in relation to high-tension glaucoma confirmed the main effect of the tagging SNP rs3918188.

Effect Modification with Age of Menopause/Postmenopausal Hormone Therapy

Because associations were observed between *NOS3* SNPs in relation to high-tension glaucoma, particularly in the women, we examined the interaction between these SNPs and attributes of reproductive aging. There were no significant interactions between any of the *NOS3* polymorphisms and age at menopause (≥54 vs. <54 years; data not shown). However, in relation to high-tension POAG, we observed significant interactions between current PMH use and four of the five *NOS3* SNPs we evaluated (Table 5). For example, among the women who were homozygous for the common allele of the T −786C SNP, current PMH use was significantly inversely associated with high-tension POAG risk (multivariate RR = 0.41; 95% CI, 0.22–0.76); however, among carriers of the minor allele, current PMH use was not associated with POAG (P-interaction = 0.04). Similar trends were observed with the tagging SNPs rs1800779 and rs7830. For the rs3918188 SNP, current PMH use was inversely associated with high-tension POAG risk only among the carriers of the minor allele (e.g., the multivariate RR of high-tension POAG among the current PMH users with the AA genotype was 0.37 [95% CI, 0.15–0.94]; P-interaction = 0.02).

Discussion

In this large, population-based, case-control study, we found no relation between *NOS3* gene variants and POAG overall.

TABLE 2. Cohort-Specific and Pooled Relative Risks of POAG

	NHS			HPFS			Pooled	
SNP/Genotype	Cases (n = 373)	Controls (n = 1082)	RR (95% CI)	Cases (n = 154)	Controls (n = 461)	RR (95% CI)	RR (95% CI)	*P*-het
Functional SNPs								
Promoter −786C/T								
T/T	137 (37.5)	414 (39.6)	1.00 (ref)	65 (42.5)	166 (36.3)	1.00 (ref)	1.00 (ref)	
C/T	166 (45.5)	458 (43.9)	1.11 (0.83–1.47)	66 (43.1)	215 (47.1)	0.94 (0.61–1.44)	1.05 (0.83–1.34)	
C/C	62 (17)	172 (16.5)	1.17 (0.81–1.70)	22 (14.4)	76 (16.6)	0.73 (0.39–1.38)	1.00 (0.64–1.55)	
			P-trend = 0.35			*P*-trend = 0.37	*P*-trend = 0.94	0.21
Glu298Asp (rs1799983)								
G/G	164 (45.2)	479 (47.0)	1.00 (ref)	72 (49.0)	203 (47.8)	1.00 (ref)	1.00 (ref)	
G/T	143 (39.4)	428 (42.0)	0.98 (0.74–1.31)	60 (40.8)	170 (40.0)	1.21 (0.77–1.89)	1.04 (0.82–1.32)	
T/T	56 (15.4)	112 (11.0)	1.45 (0.98–2.13)	15 (10.2)	52 (12.2)	1.00 (0.50–2.01)	1.33 (0.94–1.86)	
			P-trend = 0.15			*P*-trend = 0.70	*P*-trend = 0.16	0.69
Tagging SNPs								
rs1800779								
A/A	144 (38.6)	451 (41.7)	1.00 (ref)	65 (42.2)	177 (38.4)	1.00 (ref)	1.00 (ref)	
A/G	166 (44.5)	463 (42.8)	1.16 (0.87–1.53)	67 (43.5)	209 (45.3)	1.01 (0.66–1.55)	1.11 (0.88–1.40)	
G/G	63 (16.9)	168 (15.5)	1.25 (0.87–1.81)	22 (14.3)	75 (16.3)	0.78 (0.41–1.47)	1.06 (0.68–1.65)	
			P-trend = 0.19			*P*-trend = 0.55	*P*-trend = 0.62	0.23
rs3918188								
C/C	173 (46.6)	431 (40.1)	1.00 (ref)	69 (44.8)	191 (41.4)	1.00 (ref)	1.00 (ref)	
C/A	157 (42.3)	497 (46.3)	0.79 (0.60–1.03)	53 (34.4)	218 (47.3)	0.64 (0.40–1.00)	0.74 (0.59–0.94)	
A/A	41 (11.1)	146 (13.6)	0.69 (0.46–1.05)	32 (20.8)	52 (11.3)	1.50 (0.85–2.64)	0.99 (0.46–2.12)	
			P-trend = 0.04			*P*-trend = 0.58	*P*-trend = 0.56	0.10
rs7830								
C/C	172 (46.1)	461 (42.8)	1.00 (ref)	57 (41.9)	179 (43.9)	1.00 (ref)	1.00 (ref)	
C/A	146 (39.1)	499 (46.3)	0.75 (0.57–0.98)	61 (44.9)	186 (45.6)	1.17 (0.72–1.88)	0.89 (0.58–1.37)	
A/A	55 (14.8)	118 (10.9)	1.16 (0.78–1.73)	18 (13.2)	43 (10.5)	1.17 (0.59–2.31)	1.16 (0.82–1.64)	
			P-trend = 0.70			*P*-trend = 0.54	*P*-trend = 0.99	0.47

Case and control data are the number of cases (% of total group). Results are based on conditional logistic regression, with family history of glaucoma, body mass index (<22, 22–23.9, 24–25.9, 26–27.9, 28–29.9, or ≥30 kg/m^2), smoking status (current, past, or never smoker), physical activity (quartiles of activity intensity/day), self-reported history (yes/no) of hypertension and diabetes, cumulatively updated alcohol intake (0, 1–9, 10–19, 20–20, or ≥30 g/day) and cumulatively updated caffeine intake (0–149, 150–299, 300–449, 450–600, or ≥600 g/day).

However, several SNPs, including the functional T –786C SNP in the promoter region, were associated with high-tension glaucoma, particularly in the women. The interactions between *NOS3* and their sex and *NOS3* and current PMH use among the postmenopausal women for high-tension POAG suggests that the biology of the sexes and gene–environment interactions play a role in POAG pathogenesis. As this is the first study to evaluate these gene–environment interactions, these findings should be interpreted with caution and confirmed in future studies in different racial/ethnic groups.

The functional SNP Glu298Asp, which has been linked to ischemic heart disease[33] and ischemic stroke,[34] was not associated with POAG overall or with the POAG subtypes. Furthermore, polymorphisms in this SNP were not associated with POAG of either sex, and this SNP did not interact with attributes of female reproductive aging. A relation between the promoter region functional SNP (T −786C) polymorphism has been reported with coronary vasospasm in a Japanese study[35]; however, this finding has not been replicated elsewhere.

In our study, we observed associations between *NOS3* gene variants and high-tension POAG, particularly among the women. Similar associations have been found in other studies. In a study of 56 cases of familial high-tension POAG and 100 controls, Tunny et al.[17] found that a *NOS3* variant close to the functional T −786C variant was positively associated with familial high-tension POAG; the associations by sex were not provided. In another study that included 58 patients with high-tension POAG, 76 with normal-tension glaucoma, and 38 control subjects, Logan et al.[18] failed to find an association between *NOS3* allelic variants and POAG overall, but did find an association between *NOS3* allelic haplotypes, including the T −786C and POAG with migraine.[18] The associations by sex were also not presented, but it is known that migraine, which is characterized by a dysfunction in the vasodilatory response, is a female-predominant condition.[36]

Our results suggest that reproductive hormones play a role in modulating IOP and the risk of POAG in women. An Australian study demonstrated that the incidence of POAG in women was lower than that in men up to the sixth decade of life, implying that before menopause, women are less likely to develop POAG.[37] PMH use among postmenopausal women produces modest reductions of IOP and may enhance optic nerve blood flow.[38–44] In the present study, the percentage of POAG cases with elevated IOP at diagnosis was lower in current PMH users than in non-PMH users (56% among the users versus 71% among the nonusers), supporting the role of PMH in IOP modulation. The Rotterdam Eye Study found that early menopause was associated with an increased risk of POAG,[20] which was confirmed in the NHS.[21] Furthermore, in the NHS, current use of estrogen with progestin was associated with a reduced risk of high-tension POAG.[21]

Circulating estrogen may influence *NOS3*, and current PMH use in postmenopausal women may systemically up-

TABLE 3. Cohort-Specific and Pooled RR of High-Tension Glaucoma (IOP ≥22 mmHg)

	NHS			HPFS			Pooled	
SNPs/Genotype	Cases (*n* = 252)	Controls (*n* = 731)	RR (95% CI)	Cases (*n* = 112)	Controls (*n* = 334)	RR (95% CI)	RR (95% CI)	*P*-het
Functional SNPs								
Promoter −786C/T								
T/T	87 (35.4)	283 (40.0)	1.00 (ref)	47 (42.3)	127 (38.4)	1.00 (ref)	1.00 (ref)	
C/T	108 (43.9)	318 (44.9)	1.13 (0.79–1.62)	48 (43.3)	151 (45.6)	1.06 (0.63–1.77)	1.10 (0.82–1.48)	
C/C	51 (20.7)	107 (15.1)	1.80 (1.14–2.85)	16 (14.4)	53 (16.0)	1.02 (0.48–2.17)	1.47 (0.86–2.51)	
			P-trend = 0.02*			*P*-trend = 0.89	*P*-trend = 0.12	0.24
Glu298Asp (rs1799983)								
G/G	108 (44.4)	317 (46.3)	1.00 (ref)	57 (53.8)	153 (49.8)	1.00 (ref)	1.00 (ref)	
G/T	96 (39.5)	288 (42.0)	1.01 (0.71–1.44)	40 (37.7)	117 (38.1)	1.21 (0.71–2.05)	1.07 (0.80–1.44)	
T/T	39 (16.1)	80 (11.7)	1.48 (0.91–2.40)	9 (8.5)	37 (12.1)	0.89 (0.37–2.12)	1.31 (0.85–2.01)	
			P-trend = 0.19			*P*-trend = 0.87	*P*-trend = 0.23	0.58
Tagging SNPs								
rs1800779								
A/A	93 (36.9)	307 (42.0)	1.00 (ref)	47 (42.0)	134 (40.1)	1.00 (ref)	1.00 (ref)	
A/G	107 (42.5)	322 (44.0)	1.14 (0.79–1.63)	49 (43.7)	148 (44.3)	1.13 (0.68–1.88)	1.13 (0.85–1.52)	
G/G	52 (20.6)	102 (14.0)	1.87 (1.19–2.94)	16 (14.3)	52 (15.6)	1.11 (0.52–2.36)	1.58 (0.97–2.55)	
			P-trend = 0.01*			*P*-trend = 0.68	*P*-trend = 0.02	0.31
rs3918188								
C/C	128 (51.2)	283 (39.0)	1.00 (ref)	47 (42.0)	137 (41.0)	1.00 (ref)	1.00 (ref)	
C/A	97 (38.8)	343 (47.2)	0.61 (0.43–0.85)	39 (34.8)	158 (47.3)	0.58 (0.34–1.02)	0.60 (0.45–0.80)	
A/A	25 (10.0)	100 (13.8)	0.48 (0.28–0.82)	26 (23.2)	39 (11.7)	1.48 (0.77–2.84)	0.83 (0.28–2.48)	
			P-trend = 0.0008*			*P*-trend = 0.61	*P*-trend = 0.48	0.02
rs7830								
C/C	121 (48.2)	308 (42.3)	1.00 (ref)	41 (41.0)	138 (46.2)	1.00 (ref)	1.00 (ref)	
C/A	93 (37.1)	343 (47.1)	0.66 (0.47–0.92)	43 (43.0)	129 (43.1)	1.28 (0.71–2.29)	0.88 (0.46–1.66)	
A/A	37 (14.7)	77 (10.6)	1.16 (0.70–1.92)	16 (16.0)	32 (10.7)	1.34 (0.62–2.93)	1.21 (0.79–1.85)	
			P-trend = 0.49			*P*-trend = 0.37	*P*-trend = 0.99	0.26

Results are based on conditional logistic regression, with an additional control for the covariates specified in the footnote of Table 2.

* *P*-heterogeneity was <0.05 for the test between high-tension glaucoma and normal-tension glaucoma only among NHS participants (*P* = 0.02 for the −786 C/T SNP, *P* = 0.02 for the rs3918188 SNP, and *P* = 0.04 for the rs1800779 SNP).

regulate *NOS3*. Yang et al.[45] found that estrogen enhances the release of NO from cultured human coronary artery endothelial cells. Furthermore, brachial artery vasoconstriction was documented within 1 week of surgical menopause, supporting the role of estrogen in modulating endothelial NOS activity.[46] In postmenopausal women not treated with PMHs, the endothelial NOS immunoreactivity in the uterine wall was significantly lower when compared to tissue from PMH users.[47] Perara et al.[48] found that small arteries from patients with type 2 diabetes exhibited enhanced vascular relaxation ex vivo after 6 months of PMH exposure. Presumably, these changes are mimicked in other tissue beds, including the eye, and these data support the interaction between *NOS3* variants and PMH in relation to high-tension POAG.

Limitations of our study should be considered. First, our definition of glaucoma was based on self-report and confirmation with medical records and visual fields. This definition has very high specificity, as we required documentation of reproducible defect on at least two reliable visual field tests. It is possible that given the insidiousness of glaucoma, some control subjects may have had undiagnosed glaucoma. However, it is unlikely to have had a major influence on our results, as the prevalence of glaucoma in adults over age 40 is 1.3% in Caucasians.[49] Furthermore, our control subjects had to have undergone an eye examination as of the matched cases' diagnosis dates, and the average number of eye examinations reported as of their selection as controls was three, implying that advanced glaucoma, if present, would most likely have been detected. Any misclassifications of the disease in the control group or in the POAG group would have biased the results toward the null. Third, our participants were generally healthy Caucasians, and thus we are unable to make inferences to less healthy or minority populations. Finally, it is possible that our results may be due to chance, as several *NOS3* polymorphisms as well as several interactions were examined in relation to three outcomes. Therefore, these findings should be confirmed in future studies, particularly with different racial/ethnic groups, and the results should be interpreted with caution.

To summarize the major findings of this study, we observed the following:

- None of the *NOS3* SNPs investigated was significantly associated with overall POAG
- For high-tension POAG, the association with one tagging SNP (rs3918188) was significantly inversely associated among the women but not among the men (*P*-heterogeneity by sex = 0.02).
- In the women, four of the five *NOS3* SNPs showed significant interactions with current PMH use in relation to high-tension POAG. For example, PMH use was inversely associated (RR = 0.41; 95% CI, 0.22–0.76) only among the women with the TT genotype in T −786C.

In conclusion, *NOS3* gene polymorphisms may interact with sex and PMH use in women in relation to high-tension POAG, suggesting that sex-based biology and reproductive hormones play a role in POAG pathogenesis.

TABLE 4. Cohort-Specific and Pooled RR of Normal-Tension Glaucoma (IOP <22 mm Hg)

	NHS			HPFS			Pooled	
SNP/Genotype	Cases (*n* = 121)	Controls (*n* = 351)	RR (95% CI)	Cases (*n* = 42)	Controls (*n* = 127)	RR (95% CI)	RR (95% CI)	*P*-het
Functional SNPs								
Promoter −786C/T								
T/T	50 (42.0)	131 (39.0)	1.00 (ref)	18 (42.9)	39 (31.0)	1.00 (ref)	1.00 (ref)	
C/T	58 (48.7)	140 (41.7)	1.05 (0.62–1.76)	18 (42.9)	64 (50.8)	0.44 (0.15–1.27)	0.77 (0.34–1.74)	
C/C	11 (9.3)	65 (19.3)	0.48 (0.22–1.02)	6 (14.2)	23 (18.2)	0.28 (0.05–1.49)	0.44 (0.22–0.87)	
			P-trend = 0.11*			*P*-trend = 0.08	*P*-trend = 0.03	0.32
Glu298Asp (rs1799983)								
G/G	56 (46.6)	162 (48.5)	1.00 (ref)	15 (36.6)	50 (42.4)	1.00 (ref)	1.00 (ref)	
G/T	47 (39.2)	140 (41.9)	1.02 (0.61–1.70)	20 (48.8)	53 (44.9)	1.04 (0.34–3.18)	1.02 (0.64–1.63)	
T/T	17 (14.2)	32 (9.6)	1.56 (0.75–3.24)	6 (14.6)	15 (12.7)	3.75 (0.74–19.08)	1.81 (0.93–3.52)	
			P-trend = 0.34			*P*-trend = 0.21	*P*-trend = 0.17	0.45
Tagging SNPs								
rs1800779								
A/A	51 (42.1)	144 (41.0)	1.00 (ref)	18 (42.9)	43 (33.9)	1.00 (ref)	1.00 (ref)	
A/G	59 (48.8)	141 (40.2)	1.16 (0.70–1.93)	18 (42.9)	61 (48.0)	0.51 (0.18–1.47)	0.88 (0.42–1.88)	
G/G	11 (9.1)	66 (18.8)	0.53 (0.25–1.14)	6 (14.2)	23 (18.1)	0.32 (0.06–1.65)	0.49 (0.25–0.97)	
			P-trend = 0.24*			*P*-trend = 0.11	*P*-trend = 0.09	0.34
rs3918188								
C/C	45 (37.2)	148 (42.5)	1.00 (ref)	22 (52.4)	54 (42.5)	1.00 (ref)	1.00 (ref)	
C/A	60 (49.6)	154 (44.3)	1.37 (0.83–2.26)	14 (33.3)	60 (47.3)	0.41 (0.15–1.12)	0.81 (0.25–2.61)	
A/A	16 (13.2)	46 (13.2)	1.37 (0.66–2.87)	6 (14.3)	13 (10.2)	1.28 (0.29–5.59)	1.35 (0.70–2.62)	
			P-trend = 0.26*			*P*-trend = 0.54	*P*-trend = 0.56	0.29
rs7830								
C/C	51 (41.8)	153 (43.7)	1.00 (ref)	16 (44.4)	41 (37.6)	1.00 (ref)	1.00 (ref)	
C/A	53 (43.4)	156 (44.6)	0.90 (0.55–1.46)	18 (50.0)	57 (52.3)	0.68 (0.21–2.24)	0.87 (0.55–1.36)	
A/A	18 (14.8)	41 (11.7)	1.10 (0.53–2.25)	2 (5.6)	11 (10.1)	0.34 (0.04–3.14)	0.98 (0.49–1.95)	
			P-trend = 0.98			*P*-trend = 0.30	*P*-trend = 0.73	0.33

Results based on conditional logistic regression, with additional control for covariates specified in the footnote of Table 2.

* *P*-heterogeneity was <0.05 for the test between high-tension and normal-tension glaucoma, only among NHS participants. $P = 0.02$ for the −786 C/T SNP, $P = 0.02$ for the rs3918188 SNP, and $P = 0.04$ for the rs1800779 SNP).

TABLE 5. Effect Modification by Current PMH Use Status on the Associations of Selected *NOS3* Polymorphisms and High-Tension Glaucoma (IOP ≥22 mmHg) among Women

	Never or Past Users of PMH			Current Users of PMH			
SNP/Genotype	Cases (*n* = 153)	Controls (*n* = 389)	RR (95% CI)	Cases (*n* = 72)	Controls (*n* = 233)	RR (95% CI)	*P*-int
Promoter −786C/T							
T/T	58 (41.7)	131 (37.7)	1.00	20 (28.2)	98 (43.2)	0.41 (0.22–0.76)	
T/C	57 (41.0)	173 (49.9)	0.78 (0.49–1.25)	31 (43.6)	93 (41.0)	0.69 (0.39–1.20)	
C/C	24 (17.3)	43 (12.4)	1.27 (0.68–2.40)	20 (28.2)	36 (16.8)	1.26 (0.62–2.55)	
							0.04
Glu298Asp (rs1799983)							
G/G	66 (47.5)	153 (45.5)	1.00	27 (39.1)	101 (46.3)	0.53 (0.30–0.93)	
G/T	51 (36.7)	145 (43.2)	0.83 (0.52–1.33)	31 (44.9)	93 (42.7)	0.73 (0.42–1.27)	
T/T	22 (15.8)	38 (11.3)	1.57 (0.82–3.01)	11 (15.9)	24 (11.0)	1.05 (0.46–2.41)	
							0.41
rs1800779							
A/A	60 (42.0)	144 (40.1)	1.00	23 (31.9)	104 (44.6)	0.48 (0.26–0.87)	
A/G	60 (42.0)	172 (47.9)	0.90 (0.57–1.42)	29 (40.3)	98 (42.1)	0.67 (0.38–1.17)	
G/G	23 (16.0)	43 (12.0)	1.26 (0.67–2.38)	20 (27.8)	31 (13.3)	1.56 (0.76–3.19)	
							0.05
rs3918188							
C/C	64 (45.4)	147 (40.8)	1.00	45 (62.5)	84 (36.4)	1.21 (0.71–2.07)	
C/A	61 (43.3)	171 (47.5)	0.83 (0.53–1.30)	20 (27.8)	110 (47.6)	0.37 (0.20–0.69)	
A/A	16 (11.4)	42 (11.7)	0.84 (0.41–1.69)	7 (9.7)	37 (16.0)	0.37 (0.15–0.94)	
							0.02
rs7830							
C/C	76 (53.5)	144 (40.0)	1.00	31 (43.1)	98 (42.2)	0.50 (0.29–0.85)	
C/A	49 (34.5)	173 (48.1)	0.48 (0.31–0.77)	29 (40.3)	112 (48.3)	0.41 (0.24–0.71)	
A/A	17 (12.0)	43 (11.9)	0.59 (0.30–1.18)	12 (16.7)	22 (9.5)	0.82 (0.35–1.90)	
							0.05

Data are based on analysis of data from postmenopausal women only, with adjustment for age at menopause and type of menopause (natural, surgery) in addition to those specified in the footnote of Table 2.

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